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Topographical Mapping of Epitopes on the Glycoproteins of Murine Hepatitis Virus-4 (Strain JHM): Correlation with Biological Activities

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Monoclonal hybridoma antibodies (MAb) of defined polypeptide specificity and biological activity were used in a competition binding assay to identify antibody binding sites (epitopes) on the glycoproteins of murine hepatitis virus-4 strain JHM (MHV-4). Individual MAb were labeled with horseradish peroxidase (HRP) and used as probes in a competition enzyme immunoassay (EIA). Four topographically distinct antigenic sites were detected on the E2 glycoprotein of MHV-4. Antibodies reacting with these four determinants provisionally designated A(E2), B(E2), C(E2), and D(E2) had corresponding biological activities (M. J. Buchmeier, H. A. Lewicki, P. J. Talbot, and R. L. Knobler (1984) *Virology* 132, 261-270). Antibodies to sites A(E2) and B(E2) mediated virus neutralization *in vitro* and passively protected mice against lethal virus challenge *in vivo*. Antibody to site C(E2) neutralized virus efficiently *in vitro* but did not alter disease *in vivo*, while antibody to site D(E2) neither neutralized nor protected. Two major nonoverlapping antigenic sites were defined on the E1 glycoprotein. Overlapping epitopes A(E1) and B(E1) constituted one site and epitope C(E1) the other.

INTRODUCTION

The JHM strain of MHV-4 is a neurotropic member of the Coronaviridae (Tyrrell *et al.*, 1975). Under appropriate conditions or by the use of attenuated temperature sensitive mutants of MHV-4, demyelination results (Weiner, 1973; Stohlman and Weiner, 1981; Knobler *et al.*, 1981, 1982) which has been investigated for parallels with human demyelinating disorders of unknown etiology (Maugh, 1977). Studies have shown that demyelination during MHV-4 infection is a direct effect of virus infection of the oligodendrocyte (Weiner, 1973; Lampert *et al.*, 1973; Knobler *et al.*, 1982), an effect normally masked during acute infection when neuronal infection results in fulminant encephalomyelitis (Weiner, 1973; Stohlman and Weiner, 1981). Despite elegant morphological studies from several laboratories (Weiner, 1973; Lampert *et al.*, 1973; Nagashima *et al.*, 1978; Sorensen *et al.*, 1980; Knobler *et al.*, 1982), detailed understanding of the factors which influence virus-induced demyelination is still lacking.

The MHV virion contains a helical nucleocapsid composed of a 5.4-6.9 $\times$ 10^6 relative molecular weight (M_r) single-stranded, message sense RNA and a 50-63 K ($M_r \times 10^{-3}$) nucleocapsid (N) protein (Siddell *et al.*, 1982; Sturman and Holmes, 1983). In addition to the N protein, the virion contains two glycoproteins termed E1 and E2 by Sturman and Holmes (1977). A structural model for the incorporation of E1 and E2 into MHV virions has recently been proposed (Sturman *et al.*, 1980; Sturman and Holmes, 1983). The large glycoprotein E2 is associated with the virion peplomers, contains N-linked oligosaccharides, and is composed of at least two related polypeptides: 150-200K and 90-100K (Sturman and Holmes, 1977; Wege *et al.*,

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1979; Bond *et al.*, 1979; Siddell, 1982). An intracellular 150K glycosylated precursor polypeptide has been described (Siddell *et al.*, 1981; Siddell, 1982) with an apoprotein of 110–120K (Rottier *et al.*, 1981). The E1 glycoprotein contains O-linked oligosaccharides (Niemann and Klenk, 1981; Holmes *et al.*, 1981) and appears to be embedded in the membrane. Available evidence suggests that E1 interacts with the internal N protein (Sturman *et al.*, 1980). In the virions, both glycosylated 23–26K and nonglycosylated 18–23K forms of the E1 protein exist (Wege *et al.*, 1979; Siddell *et al.*, 1981; Rottier *et al.*, 1981).

Monoclonal antibodies (MAb) were generated in this laboratory against the structural proteins of MHV-4. These MAb were used in previous studies to assign the attachment and fusion activities of the virus to the glycoprotein E2 (Collins *et al.*, 1982). In order to more precisely define the structure of the E1 and E2 glycoproteins, we have performed competition enzyme immunoassays to define epitopes associated with previously described biological activities of the MHV-4 glycoproteins.

MATERIALS AND METHODS

Virus and cell culture. Murine hepatitis virus-4 was originally obtained from Dr. Leslie P. Weiner (Collins *et al.*, 1982) and is routinely propagated and assayed in this laboratory on L-24 cells as previously described (Collins *et al.*, 1982). Cells were infected with MHV-4 (m.o.i. = 0.01–1) and incubated until extensive syncytium formation was observed (15–20 hr) to serve as a source of viral antigen for immunoblotting and EIA assays described below.

Monoclonal antibodies to MHV-4. The generation and characterization of MAb to MHV-4 structural proteins were described previously (Collins *et al.*, 1982). Virus neutralization assays and passive protection studies were performed as described (Buchmeier *et al.*, 1984).

Western immunoblotting. In addition to immunoprecipitation (Collins *et al.*, 1982), specificity of our MAb was defined by Western immunoblotting (Towbin *et al.*, 1979). For this technique, cytosol extracts of infected and control uninfected L-24 cells were prepared by a modification of a previously described method (Collins *et al.*, 1982). Infected and control cultures (2×10^7 cells) were washed with rinse buffer (20 m*M* Tris–HCl, pH 9.0; 137 m*M* NaCl; 1 m*M* $CaCl_2$, and 0.5 m*M* $MgCl_2$) and solubilized for 20 min in 3-ml ice-cold lysis buffer (rinse buffer with 1% (v/v) Nonidet-P40; 10% (v/v) glycerol, and 1% (v/v) aprotinin). Cell debris was pelleted at 1000 *g* for 10 min and cytosols were obtained after centrifugation at 100,000 *g* for 60 min. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 10.5% acrylamide separating gels (Laemmli, 1970). Nitrocellulose replicas were prepared by electrophoretic transfer for 6 hr at 5 V/cm as previously described (Erickson *et al.*, 1982). Quantitative transfer of even high molecular weight (>150K) proteins was achieved.

Following transfer, nitrocellulose was saturated by incubation for 60 min with 3% (w/v) bovine serum albumin in PBS with 0.5% (v/v) Nonidet-P40, 0.1% (w/v) sodium deoxycholate, and 1% (v/v) normal goat serum. Strips were incubated for 3 hr at 37° with hybridoma cell supernatants diluted fourfold with the same buffer, then washed three times in PBS + 0.05% (v/v) Tween 20. Bound antibodies were detected by incubation for 1 hr with ^{125}I-labeled, affinity-purified goat antibody to mouse Ig (Buchmeier *et al.*, 1981; sp act 3–5 μCi/μg; 10^6 cpm per gel lane). The strips were again washed, dried, and autoradiographed.

Coupling of horseradish peroxidase (HRP) to monoclonal antibodies. Immunoglobulin fractions were isolated from ascites fluids by precipitation at 4° with an equal volume of saturated ammonium sulfate (pH 7.0). After overnight dialysis against three changes of 0.01 *M* sodium carbonate buffer, pH 9.5, immunoglobulin (Ig) concentrations were estimated by radial immunodiffusion against goat anti-mouse IgG (Meloy Laboratories, Springfield, Va.). Antibodies were coupled to HRP (Sigma, St. Louis, Mo.) by the periodate method (Wilson and Nakane, 1978) and stored in an equal volume of glycerol at −70°. This technique has previously been

shown to covalently couple an average of one enzyme molecule per immunoglobulin via secondary amino groups (Wilson and Nakane, 1978).

Enzyme immunoassays (EIA). The solid-phase immunoassay principle employing microtiter plates was used throughout. Preliminary studies (Talbot and Salmi, unpublished, 1983) showed that certain subcellular fractions from infected cells could be used as antigens on solid phase. A particulate fraction was prepared from uninfected and MHV-4-infected cells as follows. Cells were scraped off the flasks, washed three times with cold Dulbecco's phosphate buffer saline, pH 7.4 (PBS), and resuspended in the same buffer (4×10^7 cells/ml). Cell suspensions were disrupted by five cycles of rapid freezing and thawing followed by two 1-min cycles of sonication on ice slurry with a Microtip at a maximum energy in a probe-type sonicator (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.). Cellular debris was removed by centrifugation at 1000 *g* for 10 min and particulate material pelleted at 100,000 *g* for 60 min. Pellets were resuspended in PBS and sonicated as above. Protein concentration was determined (Lowry *et al.*, 1951) using bovine serum albumin as a standard. Antigens were stored at 4° in the presence of 0.5 m*M* merthiolate.

Microtiter plates (Linbro Titertek, Flow Laboratories, Cat. No. 76-202-05) were coated with 0.1 ml/well of viral or control antigens with total protein concentration of 5 μg/ml. After overnight antigen coating at room temperature, free binding sites of the polystyrene wells were blocked with 0.15 ml/well of EIA diluent (PBS supplemented with 10% (v/v) heat-inactivated fetal calf serum, 0.2% (v/v) Tween 20, and 0.5 m*M* merthiolate). After an incubation of at least 1 hr at room temperature, antigen-coated plates were used for antibody binding or for competition binding assays.

In antibody binding assays, serial two-fold dilutions of monoclonal antibodies as ascites fluids were incubated in antigen-coated plates for 75 to 90 min at room temperature which was the incubation temperature in all the subsequent steps as well. The plates were washed three times with a washing solution (PBS supplemented with 0.05% (v/v) Tween 20) and HRP-labeled affinity-purified antibodies to mouse IgG and IgM (Boehringer-Mannheim Biochemicals, Indianapolis, Ind.) were added. After 75 to 90 min incubation, the plates were washed as above and 0.1 ml of the substrate solution consisting of 3 m*M* hydrogen peroxide and 2.2 m*M* *o*-phenylenediamine in 0.1 *M* sodium citrate buffer, pH 5.0 was added into each well. The enzyme reaction was terminated after 30 min incubation in subdued light by adding 0.1 ml of 1 *N* HCl into each well. Optical densities were read (OD_{492}) with a Titertek Multiskan photometer (manufactured by Eflab, Helsinki, Finland).

The competition binding assays were performed as above with the following modifications. After 75 to 90 min incubation of fourfold dilutions of the ascites samples containing different MAb, only one gentle washing cycle was performed. A previously determined optimal amount of HRP-conjugated MAb to MHV-4 was added to the plates in volumes of 0.1 ml/well. After 75 to 90 min incubation, plates were washed three times, substrate added, and OD_{492} read as above. Since the non-specific binding of individual conjugates varied considerably, binding to control antigen was taken to represent background, and binding of each conjugate to viral antigen as maximum binding. This difference was taken to represent 100% binding, and the relative inhibition by the competing first antibody was normalized to this scale in each individual test.

RESULTS

Polypeptide specificity of monoclonal antibodies to MHV-4. The polypeptide specificity of monoclonal antibodies against MHV-4 was initially characterized by immunoprecipitation of radiolabeled viral proteins from infected cell extracts (Collins *et al.*, 1982). While specificities could be assigned based upon the predominant polypeptide in immunoprecipitates, coprecipitation of other viral polypeptides was occasionally observed. These ambiguities were eliminated by the immunoblotting

technique, as illustrated in Fig. 1A. Monoclonal antibodies to the E2 glycoprotein reacted with virus-specific polypeptides migrating at approximately 100, 160, and 230K, as well as with high molecular weight aggregates which did not penetrate separating gels (Wege *et al.*, 1979; Siddell *et al.*, 1981). Antibodies to the glycoprotein E1 reacted only with a doublet of 25 and 23K. Nucleocapsid protein antibodies reacted only with a 56K protein. The summarized results of immunoblotting analyses and the corresponding biological properties are shown in Table 1. Six of thirteen antibodies to MHV-4 glycoproteins E1 and E2 were unable to bind to denatured viral polypeptides blotted to nitrocellulose whereas all of five antibodies to N bound well to the denatured molecule. For these antibodies which did not react on Western immunoblots, the previously described (Collins *et al.*, 1982) immunoprecipitation technique was used (Fig. 1B).

Four of eight antibodies to E2 neutralized virus infectivity *in vitro*, recapitulating previous results (Collins *et al.*, 1982). With one exception (antibody 4B11.6), the neutralizing antibodies were found in separate studies to be protective upon passive transfer to mice prior to or concurrent with intracerebral virus challenge (Buchmeier *et al.*, 1984).

Enzyme immunoassay of MAb to MHV-4 glycoproteins. Comparative studies indicated that a membrane-enriched fraction prepared from virus-infected cells provided the optimal antigen source and that all antibodies showed binding characteristics to the antigen preparation equivalent to their binding to purified virions (Talbot and Salmi, unpublished, 1983). The minimum antigen concentration (0.5 μg total protein per well) which provided maximum antibody binding with all of the three classes of antibodies examined was selected and used throughout this study (Fig. 2). Binding characteristics of MAb to MHV-4 glycoproteins were determined by parallel solid-phase EIA titrations using a single antigen preparation. Binding curves of

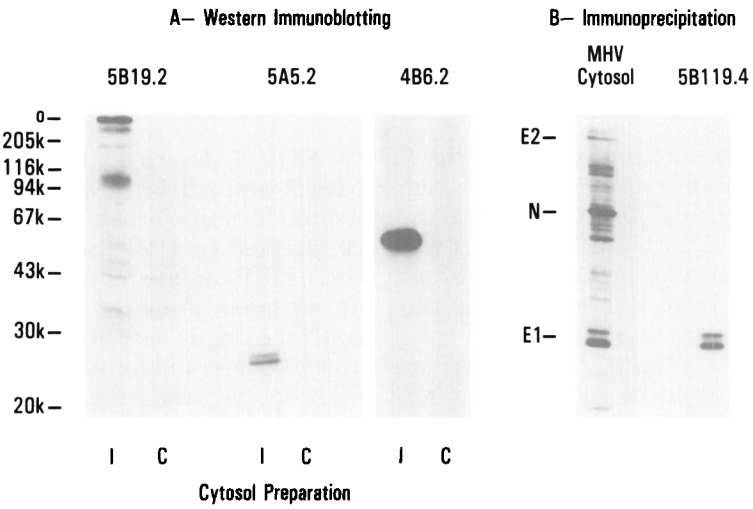

FIG. 1. Polypeptide specificities of MHV-4 monoclonal antibodies. Antibodies were reacted by Western immunoblotting (Fig. 1A) on nitrocellulose replica of SDS-PAGE-separated polypeptides from infected (I) or control (C) cytosol preparations of L-24 cells, as described in the text. Migration positions of molecular weight markers are shown on the left, as well as the origin of the separating gel (o). Monoclonal antibodies and their polypeptide reactivities were 5B19.2 (anti-E2: 100, 160, 230K, and high molecular weight aggregates at the origin), 5A5.2 (anti-E1: 23K, 25K) and 4B6.2 (anti-N: 56K). Immune precipitation of viral polypeptides from cytosol of MHV4-infected L-24 cells by monoclonal antibodies (Fig. 1B). Cells were infected with MHV-4, then pulse-labeled with [^{35}S]methionine and a cytosol extract prepared and immunoprecipitated with antibody 5B119.4 specific for the E1 polypeptides 23 and 25K.

TABLE 1

SUMMARY OF CHARACTERISTICS OF MONOCLONAL ANTIBODIES TO MHV-4

Hybridoma designation	Ig[a] subclass	Polypeptide specificity	Reaction in Western immunoblotting[b]	*In vitro* neutralization[c]	*In vivo* protection[d]
5B19.2	IgG1	E2	+	+	+
5B170.3	IgG1		+	+	+
5A13.5	IgG2A		−	+	+
4B11.6	IgG2A		−	+	−
5B21.5	IgG1		+	−	−
5B93.9	IgA		+	−	−
5B207.7	IgG2B		+	−	−
5B216.8	IgG2A		−	−	−
5B11.5	IgG2A	E1	−	−	−
5A5.2	IgG3		+	−	−
5B119.4	IgG2A		−	−	−
5B128.3	IgG3		+	−	nd[e]
5B39.1	IgG3		−	−	nd
4B6.2	IgG1	N	+	−	−
5B145.5	IgA		+	nd	nd
5B175.6	IgG2A		+	−	nd
5B86.2	IgG3		+	nd	nd
5B188.2	IgG2A		+	nd	nd

[a] Abbreviations: Ig, immunoglobulin; nd, not done.
[b] Binding of monoclonal antibody on nitrocellulose replica of SDS-PAGE-separated polypeptides.
[c] +: 50% plaque reduction at ascites dilution of more than 1:1000 (Buchmeier *et al.*, 1984).
[d] +: >80% mice protected from lethal virus challenge (Buchmeier *et al.*, 1984).

typical antibodies are presented in Fig. 3. Most antibodies appeared to bind with high avidity to the viral glycoproteins since the maximal level of measurable binding was reached (Frankel and Gerhard, 1979; Stone and Nowinski, 1980; Massey and Schochetman, 1981).

Molecular topography of epitopes on MHV-4 glycoproteins by competition binding EIA. Our initial attempts to define the topography of antigenic sites on the MHV-4 glycoproteins made use of conventional solid-phase competitive radioimmunoassay with radioiodinated MAb. However, the binding efficiencies of many of the purified MAb were diminished following radiolabeling. On the other hand, most of our MAb retained their binding properties when conjugated to peroxidase (Wilson and Nakane, 1978), thus allowing the use of a sensitive enzyme-linked immunoassay format. Monoclonal antibodies to MHV-4 described in Table 1 (except 5B86.2 and 5B188.2, which were not yet available in the form of ascites fluids) were conjugated to HRP. Several dilutions of these HRP conjugates were titrated by EIA on antigens prepared from infected and control cells, as described under Materials and Methods, and for each conjugate, three dilutions representing approximately 90, 50, and 30% saturation of viral antigen were selected. Binding inhibition by nonconjugated homologous and heterologous antibodies was titrated against each of the three conjugate dilutions to determine the most sensitive experimental conditions.

The results of typical competition binding assays are presented in Figs. 4 and 5 and the data summarized in Table 2. Binding of HRP-conjugated MAb was inhibited from 70 to 99% in the presence of excess unlabeled homologous antibody. Variation observed related to the quality of HRP

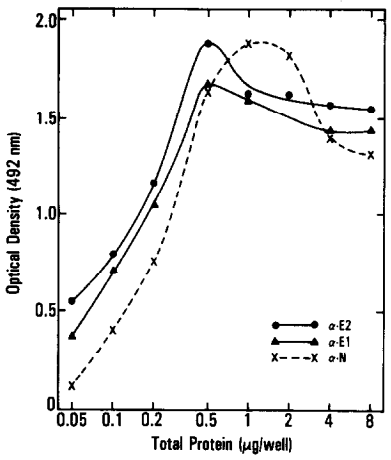

FIG. 2. Specific binding of monoclonal antibodies to a membrane-enriched fraction of MHV-4 infected cells. Various quantities of membrane-enriched fraction from MHV-4 infected or control L-24 cells were adsorbed in the wells of microtiter plates. Previously determined optimal dilutions of monoclonal ascites fluids against viral protein E2, E1, or N were bound and detected with HRP-labeled goat anti-mouse Ig, as described in the text. Specific binding was expressed as OD_{492} (virus antigen)-OD_{492} (control antigen).

conjugates obtained for each MAb. Conjugates with lower EIA titers were added at higher concentration and their binding could be inhibited to a lesser extent by unconjugated homologous antibodies, thus decreasing the sensitivity of the assay. For that reason, some conjugates did not provide reliable probes in competition binding assays. Nevertheless, all the available antibodies were tested as unconjugated competing antibodies. Figure 4D shows the absence of nonspecific blocking in our competition binding assay since none of the glycoprotein antibodies inhibited the binding of an antibody probe to the N protein.

Two major antigenic regions could be distinguished on the small glycoprotein E1 (Fig. 4). One site, designated C(E1), was defined by the monoclonal antibody 5B128.3. None of the other MAb tested (five against E1, eight against E2, and three against N) blocked the binding of the probe; only homologous inhibition was observed (Fig. 4C). Another site was defined by E1 antibodies 5B119.4, 5A5.2, and 5B11.5, which showed intermediate levels of reciprocal competition (Figs. 4A and B). This intermediate blocking could indicate structurally close and possibly overlapping epitopes which generated mutual incomplete steric hindrance upon antibody binding. Hence, we assigned epitope A(E1) to the MAb 5B119.4 and epitope B(E1) to 5A5.2 to stress this uncertainty. The target epitope of 5B11.5 could not be precisely assigned since reliable HRP-conjugated probes of this antibody were not obtained and inhibition of both 5B119.4 and 5A5.2 conjugates was observed.

Four antigenic sites could be assigned to the large glycoprotein E2 (Fig. 5). Site A(E2) was defined by two antibodies, 5B19.2 and 5B170.3, which could compete with each other for binding to the viral

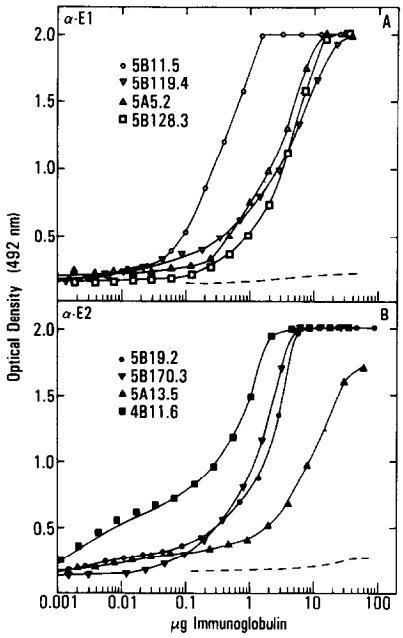

FIG. 3. Binding characteristics of different representative monoclonal antibodies to glycoproteins E1 (A) and E2 (B). Serial twofold dilutions of antibody in the form of ascites fluid were incubated in the wells of microtiter plates containing adsorbed membrane-enriched fraction from MHV-4 infected (——) or control (- - -) L-24 cells. Control curves were obtained for each antibody and showed minimal deviations from the mean curve shown here. Bound antibodies were detected with HRP-labeled goat anti-mouse Ig, as described in the text. Immunoglobulin concentration (μg) represents the total amount of unconjugated antibodies added.

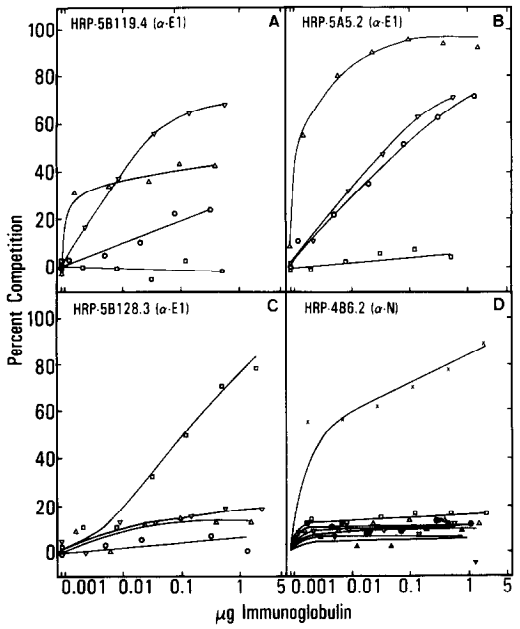

FIG. 4. Competition binding EIA with HRP-conjugated monoclonal antibodies directed against glycoprotein E1 (A-C) and nucleocapsid protein N (D). Serial fourfold dilutions of unconjugated antibodies in the form of ascites fluids were allowed to react with the antigen-coated plate. Unbound antibodies were removed and the binding of a previously determined limiting amount of HRP-conjugated monoclonal antibody probe was observed. The extent of blocking by unconjugated antibodies of conjugated probe binding was calculated as described in the text and expressed as percent competition. Immunoglobulin concentration (μg) represents the total amount of unconjugated antibodies added. Enzyme-conjugated antibodies directed against E1 were 5B119.4 (A), 5A5.2 (B), and 5B128.3 (C). Antibody 4B6.2 (D) is directed against the N protein. ○, 5B11.5; ▽, 5B119.4; △, 5A5.2; □, 5B128.3; ×, 4B6.2; ●, 5B19.2; ▼, 5B170.3; ▲, 5A13.5; ■, 4B11.6.

glycoprotein (Figs. 5A and B). Sites B and C(E2) were respectively defined by antibodies 5A13.5 and 4B11.6 and only homologous blocking was observed at those antigenic determinants (Figs. 5C and D). Site D(E2) was defined by the four nonneutralizing antibodies which could not compete for antibody binding at sites A, B, or C(E2) (data not shown).

DISCUSSION

In this study, 13 monoclonal antibodies directed against the structural glycoproteins of MHV-4 were used in competition binding immunoassays to identify antigenic regions on these glycopeptides (Yewdell and Gerhard, 1981). Based on our data and biological activities of the monoclonal antibodies studied, we were able to associate specific properties to these epitopes. Three nonoverlapping antigenic sites designated A(E2), B(E2), and C(E2) were defined on the E2 glycoprotein by four neutralizing antibodies. Sites B(E2) and C(E2) were identified by the binding of 5A13.5 and 4B11.6, respectively. Two antibodies, 5B19.2 and 5B170.3, were both directed to site A(E2). Each competing pair was examined by reciprocal competition assays with both members in a combination conjugated in turn to minimize the effect of differing avidity (Stone and Nowinski, 1980). These results presented an interesting paradox. Antibody 4B11.6 directed against site C(E2) neutralized virus efficiently *in vitro* but failed in other studies to protect mice against a lethal virus chal-

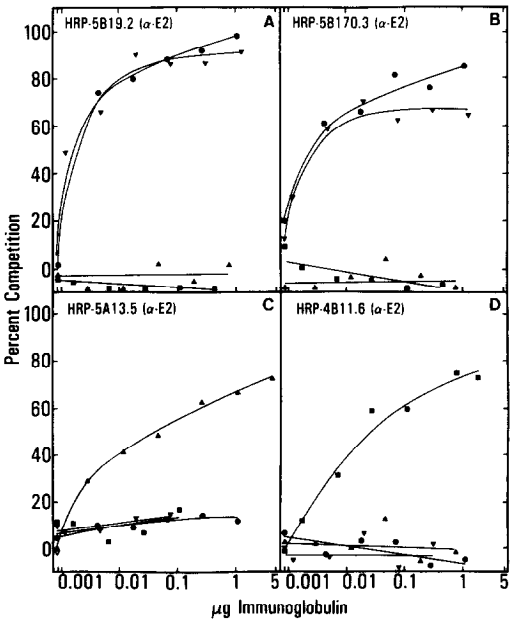

FIG. 5. Competition binding EIA with HRP-conjugated monoclonal antibodies directed against glycoprotein E2. Assays were done as described in the legend for Fig. 4. Enzyme-conjugated antibodies directed against E2 were ●, 5B19.2 (A); ▼, 5B170.3 (B), ▲, 5A13.5 (C); and ■, 4B11.6 (D).

TABLE 2

SUMMARY OF COMPETITION BINDING ENZYME IMMUNOASSAYS BETWEEN MONOCLONAL ANTIBODIES TO MHV-4[a]

Nonconjugated competing antibody	Enzyme-conjugated probe						
	E2 Antibodies				E1 Antibodies		
	5B19.2	5B170.3	5A13.5	4B11.6	5B119.4	5A5.2	5B128.3
E2 antibodies							
5B19.2	●	●	○	○	○	○	○
5B170.3	●	●	○	○	○	○	○
5A13.5	○	○	●	○	○	○	○
4B11.6	○	○	○	●	○	○	○
E1 antibodies							
5B11.5	○	○	○	○	◑	◑	○
5B119.4	○	○	○	○	●	◑	○
5A5.2	○	○	○	○	◑	●	○
5B128.3	○	○	○	○	○	○	●

Note. Summary of data presented in Figs. 4 and 5.

[a] Percentage of homologous blocking at 1 μg immunoglobulin. ●, >80%; ◑, 35-70%; ○, <30%.

lenge *in vivo* (Buchmeier *et al.*, 1984), whereas three other neutralizing antibodies, 5B19.2, 5B170.3, and 5A13.5, were protective *in vivo*. These MAb were directed to two topographically distinct antigenic sites designated A(E2) and B(E2). This indicates the presence on E2 of multiple topographically distinct regions associated with virus attachment on the host cell. Preliminary studies indicate that antigenic variation at sites B(E2) and C(E2) can be detected in various MHV strains (Talbot and Buchmeier, unpublished, 1984). These two antigenic sites may be conformation-dependent determinants since their reactivity is lost after SDS denaturation and Western immunoblotting (Table 1). We have made the assumption that antigenic sites A, B, and C(E2), all of which give rise to neutralizing antibodies, are freely accessible to the external milieu.

Four nonneutralizing E2 antibodies were shown by solid-phase EIA to bind to the viral protein, at a distinct site or sites designated D(E2) since they did not affect antibody binding to antigenic sites A, B, or C(E2). More definitive characterization awaits development of reliable conjugates of these antibodies. Binding curves for these nonneutralizing antibodies were similar to those of the neutralizing antibodies suggesting that failure to neutralize was not due to gross differences in titer or binding avidity. Nonneutralizing antibodies to viral attachment proteins have now been demonstrated for the retroviruses (Massey and Schochetman, 1981; Bruck *et al.*, 1982), togaviruses (Roehrig *et al.*, 1980; Kimura-Kuroda and Yasui, 1983), orthomyxoviruses (Breschkin *et al.*, 1981; Carter *et al.*, 1982), rhabdoviruses (Volk *et al.*, 1982), and reoviruses (Burstin *et al.*, 1982). We found that antibodies to E1 or E2 which failed to neutralize *in vitro* also did not alter the disease process *in vivo* (Buchmeier *et al.*, 1984), in contrast to nonneutralizing MAb to Sindbis virus which prevented lethal encephalitis *in vivo* (Schmaljohn *et al.*, 1982).

Glycoprotein E1 contained at least three epitopes, designated A(E1), B(E1), and C(E1), defined by five MAb. These epitopes could be grouped into two nonoverlapping antigenic sites, as defined by Yewdell and Gerhard (1981). These antibodies appear to recognize the apoprotein of E1 since they bound well to both the 23K nonglycosylated precursor and the 25K glycosylated form. Site C(E1) was clearly distinct and recognized only by antibody 5B128.3. Definition of possibly overlapping epitopes A(E1) and B(E1) by antibodies 5B119.4, 5A5.2, and

5B11.5 is less certain. Binding of these antibodies was only partially cross-inhibited and this may be due to steric hindrance rather than site specific competition as E1 is small (25K) relative to the IgG peroxidase probe (200K). Antibody 5B39.1 was shown to bind to the viral protein but did not affect the binding of the other antibodies. Its target epitope is thus likely distinct from epitopes A and B and C(E1), although the absence of a reliable enzyme conjugate of 5B39.1 did not allow its definitive topographical assignment.

Our studies further confirm the presence in infected cells of an approximately 150K precursor to the structural glycoprotein E2 (Wege *et al.*, 1979; Siddell *et al.*, 1981). Monoclonal antibodies to E2 also recognized a 100K monomer and high M_r polypeptides as reported previously (Wege *et al.*, 1979; Siddell *et al.*, 1981; Sturman and Holmes, 1983).

Studies of others on detergent-solubilized virions as well as the observation that approximately 80% of the E1 protein is protected from protease digestion have implied that E1 is a transmembranous protein interacting inside the virion with the nucleocapsid and may organize sites of virus maturation in the cell (Sturman and Holmes, 1977; Wege *et al.*, 1979; Sturman *et al.*, 1980; Holmes *et al.*, 1981). In contrast, E2 was completely digested by protease treatment and did not appear to be necessary for virus maturation since virus particles formed in the presence of tunicamycin lacked E2 peplomers (Holmes *et al.*, 1981). Recently, E2 was shown to contain covalently attached fatty acid (Schmidt, 1982), which is the only indication that a portion of this glycoprotein might be exposed to a hydrophobic environment. Therefore, current knowledge of MHV structure is consistent with the hypothesis that E1 may serve to anchor the E2 peplomers within the viral envelope (Sturman *et al.*, 1980). The apparent stoiechiometric excess of E1 over E2 proteins (Sturman *et al.*, 1980; Sturman and Holmes, 1983) would be explained if several E1 molecules formed a complex around an E2 stalk peripherally embedded in the membrane. This association would allow E2 to form hydrophobic domains by interaction with this E1 anchor. However, we have failed so far to obtain MAb directed against epitopes on the two viral glycoproteins which would be in close enough proximity to cause mutual steric hindrance in competition binding EIA. Our membrane-enriched antigen source presumably contains both plasma membranes and microsomal membranes thus obviating any speculation about the localization of the viral glycoproteins in this system. All competition binding EIA were consistent in the presence or absence of the nonionic detergent Tween 20 in the assay buffers. This eliminates the possibility of structural reorganizations induced by the detergent. Obviously, more precise structural studies are needed to define the molecular arrangement of these proteins.

Monoclonal antibodies have proven to be sensitive probes to study the biology and structure of MHV-4 and we have used them to characterize biologically important antigenic regions of the viral glycoproteins. Studies are currently underway in our laboratory to examine the structural properties of these glycoproteins with the goal of better understanding the molecular basis of virus-induced demyelination.

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